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Lack of Hepatitis C Virus Replication Intermediate RNA in Diseased Skin Tissue of Chronic Hepatitis C Patients

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The extent of extrahepatic hepatitis C virus (HCV) replication seems to be low-level and confined to cells of hematopoietic lineage. However, given the spectrum of extrahepatic manifestations associated with HCV, several tissues other than the liver have been suggested as targets of HCV replication and damage. The presence and level of HCV RNA were examined in 19 skin tissue samples from patients chronically infected with HCV and referred for lichen ruber planus (n = 11) or cutaneous vasculitis associated with mixed cryoglobulinemia (n = 8). Serum HCV RNA was quantitated and genotyped by assays that are available commercially. Tissue HCV RNA of genomic- and minus-strand polarity was titrated by a strand-specific semiquantitative RT-PCR. Low titers of genomic-strand HCV RNA were found in three skin specimens from patients with cutaneous vasculitis due to mixed cryoglobulinemia, but in none with lichen ruber planus. The replication intermediate HCV RNA was not detected in any of the skin tissues examined, independent of the serum HCV RNA level or genotype. It is concluded that the occurrence of cutaneous vasculitis and lichen ruber planus in chronic hepatitis C patients is unlikely to be due to HCV replication in the skin. ***J. Med. Virol. 59:277–280, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis C; lichen ruber planus; mixed cryoglobulinemia; immunopathogenesis

INTRODUCTION

A significant proportion of the morbidity associated with the hepatitis C virus (HCV) infection is attributable to extrahepatic manifestations [Hadziyannis, 1997]. Among these, skin diseases have received considerable attention, as they may be observed frequently in chronic hepatitis C patients [Pawlotsky et al., 1995; Hadziyannis, 1998]. The association between chronic HCV infection and cutaneous vasculitis due to the deposition of cryoglobulins is well established [Lunel, 1994]. Other skin diseases have been found in both acute and chronic hepatitis C patients; these include lichen ruber planus, psoriasis, erythema nodosum, urticaria, erythema multiforme, Behçet disease, and the cutaneous manifestations of porphyria cutanea tarda [Hadziyannis, 1998]. The pathogenesis of these conditions is sometimes controversial but is believed to be mediated mostly by the host immune system, by immune complex deposition, or by autoimmune reactions. In a previous study, Agnello and Ábel [1997] were unable to detect the minus-strand HCV RNA in cutaneous vasculitis tissue by a relatively insensitive nonradioisotope in situ hybridization, and concluded that HCV RNA in the skin is either due to virion deposition in immune complexes or passive accumulation in keratinocytes by endocytosis. In another report [Sansonno et al., 1995], HCV antigens (possibly trapped in immune complexes) were detected by immunohistochemistry only in the vessel wall of skin of patients with mixed cryoglobulinemia. Direct evidence of HCV replication in the affected tissue by a sufficiently sensitive assay is lacking, however.

A semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was developed that specifically detects the HCV RNA of minus-strand polarity, i.e., the putative replication intermediate [Negro et al., 1998]. This assay can determine the presence and level of HCV replication intermediate RNA in any given tissue with a lower detection limit of 15 copies of HCV RNA per 100 ng of total tissue RNA and in a genotype-independent manner. It has been used suc-

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cessfully to evaluate of HCV replication both in the liver [Negro et al., 1998] and in some extrahepatic tissues [Ascoli et al., 1998; Rubbia-Brandt et al., 1998]. This procedure was applied to assess the presence of genomic- and minus-strand HCV RNA in a series of skin tissue specimens taken from patients with chronic hepatitis C and lichen ruber planus or cutaneous vasculitis associated with mixed cryoglobulinemia.

MATERIALS AND METHODS

Patient Population

Between June 1996 and June 1997, 19 patients with histologically confirmed HCV-related chronic liver disease were admitted at the Division of Dermatology of our hospital because of lichen ruber planus (LRP) (11 cases) or cutaneous vasculitis associated with mixed cryoglobulinemia (8 cases). There were 7 males and 12 females; the mean age was 61.6 years (range 40–82 years) and was comparable for patients with LRP (58.4 ± 10.4) and cutaneous vasculitis (66 ± 9.9).

Serum Assays

Serum samples were stored at −80° C until use. Assays included measurement of the alanine aminotransferase (ALAT) activity, detection of antibodies to HCV structural and nonstructural proteins (by a second-generation enzyme-linked immunosorbent assay [ELISA] [Ortho Diagnostics, Raritan, NJ]), detection of the HCV RNA by a qualitative nested RT-PCR with a lower sensitivity limit of 50–200 genome-equivalents/ml according to a protocol that was described previously [Shindo et al., 1991], and by a quantitative signal amplification-based branched DNA assay (Quantiplex™ version 2, Chiron Corp., Emeryville, CA) and HCV genotyping by a line probe assay (INNO-LiPA, Innogenetics, Antwerp, Belgium).

Liver Histology

Liver biopsy specimens were taken for histological evaluation from all patients were formalin-fixed, paraffin-embedded, and stained with hematoxylin-eosin (H&E) and Masson's trichrome stainings. Histological diagnoses of liver disease followed internationally accepted criteria [Desmet et al., 1994].

Strand-Specific Semiquantitative RT-PCR for Tissue HCV RNA

Skin tissue specimens were taken at biopsy, snap-frozen in liquid nitrogen and stored at −70°C until use. Genomic- and minus-strand HCV RNA were determined by a semiquantitative RT-PCR [Negro et al., 1998]. Briefly, total tissue RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform procedure (Tri-Reagent, Sigma Chemical Co., St. Louis, MO) [Chomczynski and Sacchi, 1987]. Then, 100 ng of total RNA (or of one of its dilutions) was denatured for 1 min at 95C, chilled on ice, and reverse transcribed in the presence of 10 mM Tris-HCl, pH 8.9, 90 mM M KCl, 0.9 mM $MnCl_2$, 200 M of each of the four dNTPs, 3.5 U of rTth (Pharmacia Biotech, Dübendorf, Switzerland) and 15 pmoles of the complementary primer. For genomic-strand HCV RNA amplification, the antigenomic primer 274R was used, whose sequence is 5′-TCGCGACCCAACACTACTC-3′ and starts at position 274 of the genomic HCV RNA [Bukh et al., 1995]. For minus-strand HCV RNA amplification, the genomic primer 15F was used, which starts at position 15 of the genomic HCV RNA and has the sequence 5′-GGG-GGCGACACTCCACCA-3′. Both primers anneal to sequences within the 5′ noncoding region of HCV RNA, which are invariant among the most commonly encountered HCV genotypes [Bukh et al., 1995]. The reaction was allowed to proceed for 60 min at 65°C, then 10 l of 7.5 mM EDTA was added to chelate Mn^{2+} ions. For PCR, 15 pmoles of the primer of polarity opposite to that used in the RT reaction were added (i.e. primer 15F for genomic- and primer 274R for minus-strand RNA amplification, respectively), and the reaction run in a 100-μl mixture containing a final concentration of 10 mM Tris-HCl, pH 8.9, 0.1 M KCl, 1.5 mM $MgCl_2$. The PCR amplification consisted in 2 min at 94°C, followed by 40 cycles (1 min at 94°C, followed by 1 min at 62°Cand then by 1 min at 74° C), followed by a 7-min extension at 72° C. The appropriate size of the first PCR product (260 base pairs [bp]) was checked on a 1.6% agarose gel. For the second, nested PCR, an aliquot (1/10) of the first PCR was reamplified using 50 pmoles of each of the two primers 98F (5′-GAGTGTCG TGCAGCCTCCAG-3′) and 258F (5′-CTCGGCTAG-CAGTCTTGCGG-3′), which span the 161 base pairs region 98–258 of the HCV RNA, and 2.5 U of the Taq polymerase (Gibco-BRL, Life Technologies AG, Basel, Switzerland). The reaction was run for 35 cycles (1 min at 94°C, 1 m in at 55°C and 1 min at 72°C), followed by 7 min at 72° C. The sensitivity, strand specificity, reproducibility, and genotype independence of this assay have been reported in detail elsewhere [Negro et al., 1998, 1999].

Semiquantitation was achieved by carrying out a nested RT-PCR to the endpoint on 2- to 4-fold dilutions (in 10 μg/ml of *Escherichia coli* tRNA [Sigma]) of an initial amount of 100 ng of total liver RNA. Titers were expressed as the last dilution giving a visible band of the appropriate size on a 1.6% agarose gel stained by ethidium bromide.

RESULTS

All 20 patients had detectable HCV RNA in serum as determined by qualitative RT-PCR. Serum HCV RNA levels (Table I) ranged from <0.2 to 17.53 × 10^6 genome-equivalents/ml. HCV genotypes could be assigned in 17 patients, and their results are shown in Table I. Patients with LRP were infected with HCV genotype 1b (3 cases), 2a (4 cases) or 4 (1 case); three more patients had an INNO-LiPA pattern which could not be assigned to any genotype. Patients with cutaneous vasculitis secondary cryoglobulinemia had HCV genotype 1b (7 cases) or 2a (1 case). The difference of genotype distribution between the two groups failed

TABLE I. Clinical and Virological Characteristics of 19 Chronic Hepatitis C Patients With Skin Diseases

# N	Sex	Age (yr)	Liver biopsy diagnosis	Skin biopsy diagnosis	Serum HCV RNA level (gEq/ml)	Skin HCV RNA		HCV genotype
						Genomic-strand titer	Minus-strand titer	
1	f	54		LRP	<200,000	0	0	NA
2	f	53		LRP	<200,000	0	0	2a
3	m	58	Cirrhosis	LRP	708,000	0	0	1b
4	m	54		LRP	1,090,000	0	0	1b
5	f	64	Cirrhosis	LRP	<200,000	0	0	2a
6	f	52		LRP	914,000	0	0	2a
7	f	62	Cirrhosis	LRP	<200,000	0	0	NA
8	f	63		LRP	<200,000	0	0	2a
9	m	40	Severe CH	LRP	990,000	0	0	4
10	f	82		LRP	<200,000	0	0	NA
11	m	60	Cirrhosis	LRP	17,530,000	0	0	1b
12	f	61		Vasculitis	257,000	0	0	1b
13	m	77	Moderate CH	Vasculitis	<200,000	0	0	1b
14	f	55		Vasculitis	9,070,000	256	0	1b
15	f	50	Mild CH	Vasculitis	500,000	0	0	1b
16	m	73		Vasculitis	3,570,000	0	0	1b
17	f	73		Vasculitis	21,460,000	0	0	2a
18	f	74	Cirrhosis	Vasculitis	818,000	1	0	1b
19	m	65	Cirrhosis	Vasculitis	3,340,000	64	0	1b

yr, years; LRP, lichen ruber planus; gEq/ml, genome-equivalents/ml; NA, not assigned; CH, chronic hepatitis.

however to reach statistical significance ($P = 0.06$; Fisher's exact test).

A liver biopsy was obtained in nine patients: all had chronic hepatitis of varying necro-inflammatory activity, accompanied by cirrhosis in 6 cases (Table I).

Genomic-strand HCV RNA was detected in three skin biopsy specimens, all from patients with cryoglobulinemia-associated cutaneous vasculitis: these three samples lacked however minus-strand HCV RNA (Table I). All other patients examined, including the 11 patients with LRP, lacked both genomic and minus-strand HCV RNA in their skin biopsy specimens.

DISCUSSION

The presence of HCV replication intermediate RNA was sought in 19 skin specimens taken from chronic hepatitis C patients and HCV-associated skin affections. Minus-strand HCV RNA was undetectable in all samples tested, independent of the HCV replicative level (as evaluated in serum) and genotype, suggesting that, within the sensitivity limits of our RT-PCR (15 genome-equivalents per assay), HCV does not replicate in the skin. Thus, the pathogenesis of the cutaneous vasculitis and of the lichen ruber planus associated with HCV infection is unlikely to result from a cytopathic effect of the replicating virus in skin cells.

The data are in agreement with a previous study [Agnello and Ábel, 1997] that examined the presence of both HCV RNA strands in the cutaneous vasculitic lesions of patients with type II cryoglobulinemia by in situ hybridization. The latter study showed the presence of genomic-strand HCV RNA in some vessel walls, in the skin and ductal epithelium and in the vascular endothelium of inflamed (but not in normal) skin tissue, as well as the lack of minus-strand HCV RNA in the same cells. It was concluded that HCV does not replicate in the cutaneous vasculitic lesions and that these are secondary to the perivascular deposition of HCV virion-containing immune complexes. Furthermore, Agnello and Ábel [1997] suggested that genomic polarity HCV RNA may be accumulated passively in the skin epithelial cells by an endocytosis mechanism via interaction with the LDL receptor, overexpressed in inflamed skin. We could only find low titers of genomic-strand HCV RNA in three of the eight vasculitic tissues examined, suggesting that this passive accumulation of HCV virions in skin cells is low-level and probably inconsistent. It is possible that, in these three cases, the genomic-strand HCV RNA detected in the skin was caused by contamination from plasma, since the specimens were taken from patients with high levels of HCV RNA in the serum. However, skin samples from patients with even higher viremia levels (see patient 18, Table I) did not contain detectable levels of genomic- or minus-strand HCV RNA, showing that contamination from virions found in plasma at the time of skin tissue sampling is unlikely.

LRP is a benign disease characterized by keratinocytes degeneration and dense T lymphocytes infiltration of the upper dermis, sometimes involving the oral mucosa. Its association with chronic liver disease appears to be established, and a specific role for HCV has been suggested, especially from studies conducted in Italy and Japan [Hadziyannis, 1997]. The prevalence of lichen ruber planus in chronic hepatitis C patients is around 5% [Pawlotsky et al., 1994], and up to 26% of patients with oral lichen have chronic liver disease, one-third of whom were infected with HCV [Gandolfo et al., 1994]. The LRP lesions tend to correlate with prolonged duration of HCV infection [Daoud et al., 1995]: accordingly, the mean age of our LRP patients was relatively high and four of five patients who underwent a liver biopsy had a cirrhosis. Although the precise mechanism leading to skin damage is un-

known, host, rather than viral factors are thought to be important for LRP pathogenesis [Nagao et al., 1997]. The role of the immune system in the pathogenesis of LRP also seems to be suggested by the fact that the antiviral treatment (interferon-α) may occasionally result in a flareup of the skin lesions [Barreca et al., 1995; Negro et al., 1994; Protzer et al., 1993], although in anecdotal cases no aggravation or even an improvement was reported [Doutre et al., 1992].

The presence of LRP lesions does not appear to be associated with a specific HCV genotype [Lodi et al., 1997] or the level of HCV RNA in serum [Nagao et al., 1997]. Although, in the present series, genotype 2a seemed relatively more frequent among LRP patients, the low number of patients did not allow to draw any firm conclusion as to genotype association of skin lesions. As far as the HCV replication is concerned, we could not document the presence of either genomic- or minus-strand HCV RNA in any of the LRP tissues examined, and this also in patients with high HCV RNA levels in serum (patient 11, Table I). Therefore, this our study does not support HCV replication in LRP lesions, and that therefore HCV cannot be held responsible for a direct damage of the skin tissue in this condition.

The presence of HCV in extrahepatic compartments has been the object of many studies, due to the fact that extrahepatic manifestations are frequently observed in HCV-infected patients. Thus, replicative forms of HCV RNA have been found in cells of the hematopoietic lineage [Lérat et al., 1996; Bronowicki et al., 1998] and, more recently, in the pancreas, thyroid, and adrenal gland tissue of patients coinfected with the human immunodeficiency virus [Laskus et al., 1998]. Although the specificity of the strand-specific detection of minus-strand HCV RNA has been questioned, suitably modified assays are currently available that result in a more tightly specific detection of the HCV replication intermediate RNA. Detection of the minus-strand HCV RNA is therefore feasible, provides a semiquantitative assessment of the HCV replicative level in a given tissue, and allows studies between anatomoclinical features and viral replication. Although the present series of data are negative, we believe it was important to assess the presence of HCV replicative forms in diseased skin tissue: the lack of HCV replication in cutaneous vasculitis and LRP skin specimens argues in favor of an immune-mediated pathogenesis of these conditions.

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